

jc658 U.S. PTO



11/16/99

Case Docket No. P564-9035

Date November 16, 1999

ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

Sir:

Transmitted herewith for filing under 37 C.F.R. §1.53(b) is the patent application of:

Inventor(s): Josef ENDL; Peter STAHL; Winfried ALBERT; Guenther-Gerhard JUNG;
Delores J. SCHENDEL; Edgar MEINL and Klaus DORNMAIR

For: ANTIGEN-SPECIFIC, ACTIVATED T LYMPHOCYTES, DETECTION AND USE

This is a divisional of Application Serial No. 09/343,406, filed June 30, 1999, which is a continuation of Application Serial No. 08/967,242, filed November 5, 1997 which is a continuation of Application Serial No. 08/374,468 filed January 18, 1995.

☒ Specification (48 pages)

☒ 6 sheets of drawings

☒ Declaration and Power of Attorney

☒ Copy from a prior application for continuation or divisional

☒ Return Receipt Postcard

☒ Preliminary Amendment

☒ An Information Disclosure Statement with PTO-1449.

☒ Priority of German Application(s) No.(s) P 44 03 522.5 filed February 4, 1994; P44 01 629.8 filed January 20, 1994; P44 18 091.8 filed May 24, 1994 is claimed under 35 U.S.C. §119.

☒ A certified copy had been filed in application Serial No. 08/374,468, filed January 18, 1995 and was acknowledged in the Office Action of October 16, 1996 (Paper No. 16).

☒ The prior application is assigned of record to Roche Diagnostics, GmbH.

☒ Request to use previously filed sequence listing along with a paper copy of the Sequence Listing Under MPEP 2422.05

☒ A filing fee, calculated as shown below:

	(Col. 1)	(Col. 2)	Other Than A Small Entity	
FOR:	No. Filed	No. Extra	RATE	FEE
BASIC FEE				\$760
TOTAL CLAIMS	34 - 20 =	* 14	× 18 =	\$252
INDEP CLAIMS	02 - 3 =	* 00	× 78 =	0.00
= MULTIPLE DEPENDENT CLAIM PRESENTED			+260 =	0.00
				\$1,012

* If the difference in Col. 1 is less than zero, enter "0" in Col. 2

jc675 U.S. PTO
09/441061



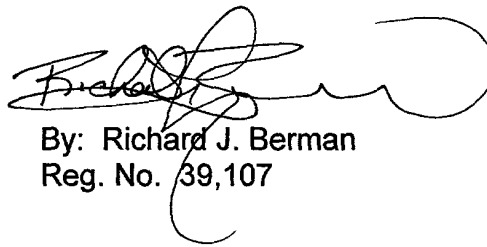
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A check in the amount of One Thousand Twelve Dollars (\$1,012.00) is enclosed to cover the filing fee. The Commissioner is hereby authorized to charge payment for any additional filing fees associated with this communication or credit any overpayment to Deposit Account No. 14-1060.

Respectfully submitted,

NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP



By: Richard J. Berman
Reg. No. 39,107

Metropolitan Square
655 Fifteenth Street, N.W.
Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000

RJB:ccd

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

ENDL, et al.

Serial Number: Not Yet Assigned

Filed: November 15, 1999

For: ANTIGEN-SPECIFIC, ACTIVATED LYMPHOCYTES, DETECTION AND USE

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

November 16, 1999

Sir:

Please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 1, after "DESCRIPTION" insert --This is a divisional of Application Serial No. 09/343,406, filed June 30, 1999, which is a continuation of Application Serial No. 08/967,242, filed November 5, 1997 which is a continuation of Application Serial No. 08/374,468 filed January 18, 1995.--

Page 3, line 23, after "Z" insert --[SEQ ID NO: 1]--.

Page 4, line 10, after "A" (final occurrence) insert --[SEQ ID NO: 2]--;
line 12, after "K" (final occurrence) insert --[SEQ ID NO: 3]--.

Page 5, line 7, after "F" (final occurrence) insert --[SEQ ID NO: 4]--.

Page 38, line 3, after "F" (final occurrence) insert --[SEQ ID NO: 4]--;
line 10, after "L" (final occurrence) insert --[SEQ ID NO: 5]--;
line 11, after "G" insert --[SEQ ID NO: 6]--;
line 12, after "A" (final occurrence) insert --[SEQ ID NO: 7]--;
line 13, after "E" (final occurrence) insert --[SEQ ID NO: 8]--;
line 14, after "L" (final occurrence) insert --[SEQ ID NO: 9]--;
line 15, after "F" (final occurrence) insert --[SEQ ID NO: 10]--;
line 16, after "S" (final occurrence) insert --[SEQ ID NO: 11]--;
line 17, after "E" insert --[SEQ ID NO: 12]--;
line 18, after "E" insert --[SEQ ID NO: 14]--;
line 19, after "T" insert --[SEQ ID NO: 14]--;
line 20, after "K" (final occurrence) insert --[SEQ ID NO: 15]--;
line 21, after "L" (final occurrence) insert --[SEQ ID NO: 16]--;
line 22, after "F" (final occurrence) insert --[SEQ ID NO: 17]--; and
line 23, after "S" (final occurrence) insert --[SEQ ID NO: 18]--.

Before the claims, add the attached Sequence Listing and renumber the pages
containing the claims accordingly.

IN THE CLAIMS:

Please delete claims 1-45 without prejudice or disclaimer and add the following new claims:

--46. A complex comprising a peptide or peptide derivative derived from glutamic acid decarboxylase which is bound to an allele or a peptide-binding derivative of MHC class II molecules DR3 or DR4, wherein the peptide or peptide derivative has a length of at most 25 amino acids and comprises

(a) a peptide of at least 6 amino acids of an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 3 and 19-39, or

(b) a peptide or peptide derivative having a length of 6 to 25 amino acids which exhibits a specificity or/and affinity which is essentially equivalent to that of the peptide (a) and includes anchor positions for binding to alleles or peptide-binding derivatives of MHC class II molecules DR3 or DR4.

47. The complex of claim 46, wherein the MHC class II molecules have the subtype DR B1 0301 or DR B1 0401.

48. The complex of claim 46, wherein the MHC class II molecules are recombinant MHC class II molecules.

49. The complex of claim 46, wherein the peptide or peptide derivative is bound to a soluble peptide-binding derivative of MHC class II molecules DR3 or DR4.

50. The complex of claim 46, wherein the complex carries a marker group.

51. The complex of claim 46, wherein the peptide (a) includes anchor positions for binding to alleles or peptide-binding derivatives of MHC class II molecules DR3 or DR4.

52. The complex of claim 46, wherein the peptide or peptide derivative comprises
(a) a peptide of at least 6 amino acids of an amino acid sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 3, or

(b) a peptide or peptide derivative having a length of 6 to 25 amino acids which exhibits a specificity or/and affinity which is essentially equivalent to that of the peptide (a) and includes anchor positions for binding to alleles of MHC class II molecules DR3 or DR4.

53. The complex of claim 46, wherein the peptide or peptide derivative comprises at least 8 amino acids.

54. The complex of claim 46, wherein the peptide or peptide derivative comprises at least 10 amino acids.

55. The complex of claim 46, wherein the peptide or peptide derivative carries a marker group.

56. A pharmaceutical composition, comprising a complex as claimed in claim 46, in combination with a pharmaceutically acceptable carrier.

57. The pharmaceutical composition of claim 56, further comprising an accessory stimulating component.

58. The pharmaceutical composition of claim 57, wherein the accessory stimulating component is a cytokine, surface antigen B7, or both.

59. A method of treating or preventing an autoimmune disease in a patient in need of such treatment or prevention, comprising administering to the patient a disease-treating or disease-preventing, respectively, effective amount of a complex as claimed in claim 46.

60. A method of treating or preventing diabetes in a patient in need of such treatment or prevention, comprising administering to the patient a disease-treating or disease-preventing, respectively, effective amount of a complex as claimed in claim 46.

61. A method of causing an immune response or an immune tolerance in a patient, comprising administering to the patient an immune response-causing or immune tolerance-causing, respectively, amount of a complex as claimed in claim 46.

62. An oligomerized peptide or peptide derivative/MHC molecule or MHC molecule derivative complex comprising (1) at least one peptide or peptide derivative and (2) at least two MHC molecules or peptide-binding MHC molecule derivatives, wherein the

at least two MHC molecules or MHC molecule derivatives are (a) directly linked to each other, wherein the at least one peptide or peptide derivative is linked to at least one of the at least two MHC molecules or MHC molecule derivatives, or (b) indirectly linked to each other via a direct or indirect linking to the at least one peptide or peptide derivative.

63. The oligomerized complex of claim 62, wherein the oligomerized complex comprises a plurality of chemically coupled peptide or peptide derivative/MHC molecule or MHC molecule derivative complex monomers.

64. The oligomerized complex of claim 62, wherein the at least one peptide or peptide derivative contains at least two MHC-binding regions and the at least two MHC molecules or MHC molecule derivatives are bound to the peptide or peptide derivative via the MHC-binding regions.

65. The oligomerized complex of claim 64, wherein the at least two MHC-binding regions are indirectly linked to each other and are separated from one another on the at least one peptide or peptide derivative by a spacer region.

66. The oligomerized complex of claim 65, wherein the spacer region comprises 10-15 amino acids.

67. The oligomerized complex of claim 62, wherein the oligomerized complex comprises a plurality of peptide or peptide derivative/MHC molecule or MHC molecule derivative complex monomers which are linked to each other via antibodies.

68. The oligomerized complex of claim 62, wherein the at least two MHC molecules or MHC molecule derivatives are MHC class II molecules or derivatives thereof.

69. The oligomerized complex of claim 68, wherein the MHC class II molecules or derivatives thereof are MHC class II molecules DR3 or DR4 or derivatives thereof.

70. The oligomerized complex of claim 69, wherein the MHC class II molecules DR3 or DR4 or derivatives thereof have the subtype DR B1 0301 or DR B1 0401.

71. The oligomerized complex of claim 62, wherein the at least two MHC molecules or MHC molecule derivatives are recombinant molecules.

72. The oligomerized complex of claim 62, wherein the oligomerized complex comprises at least one soluble peptide-binding MHC molecule derivative.

73. The oligomerized complex of claim 62, wherein the at least one peptide or peptide derivative has a length of at most 25 amino acids and comprises

(a) a peptide of at least 6 amino acids of an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 3 and 19-39, or

(b) a peptide or peptide derivative having a length of 6 to 25 amino acids which exhibits a specificity or/and affinity which is essentially equivalent to that of the peptide (a) and includes anchor positions for binding to alleles or peptide-binding derivatives of MHC class II molecules DR3 or DR4.

74. A pharmaceutical composition, comprising an oligomerized complex as claimed in claim 62, in combination with a pharmaceutically acceptable carrier.

75. The pharmaceutical composition of claim 74, further comprising an accessory stimulating component.

76. The pharmaceutical composition of claim 75, wherein the accessory stimulating component is a cytokine, surface antigen B7, or both.

77. A method of treating or preventing an autoimmune disease in a patient in need of such treatment or prevention, comprising administering to the patient a disease-treating or disease-preventing, respectively, effective amount of an oligomerized complex as claimed in claim 62.

78. A method of treating or preventing diabetes in a patient in need of such treatment or prevention, comprising administering to the patient a disease-treating or disease-preventing, respectively, effective amount of an oligomerized complex as claimed in claim 62.

79. A method of causing an immune response or an immune tolerance in a patient, comprising administering to the patient an immune response-causing or immune tolerance-causing, respectively, amount of an oligomerized complex as claimed in claim 62.--

REMARKS

This is a divisional of Application Serial No. 09/343,406, filed June 30, 1999, which is a continuation of Application Serial No. 08/967,242, filed November 5, 1997 which is a continuation of Application Serial No. 08/374,468 filed January 18, 1995.

Should any additional fees be due with respect to this paper, such fees may be charged to Counsel's Deposit Account No. 14-1060.

Respectfully submitted,
NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP



Richard J. Berman
Attorney for Applicants
Registration No. 39,107

Atty. Docket No. P564-9035
Metropolitan Square
655 Fifteenth Street, N.W.
Suite 330 - G-Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000

RJB:ccd

Boehringer Mannheim GmbH
Sandhofer Straße 112-132
68305 Mannheim-Waldhof

Antigen-specific, activated T lymphocytes,
detection and use

DESCRIPTION

The present invention concerns peptides which cause an autoimmune reaction, complexes of these peptides with molecules of the major histocompatibility complex (MHC), T cell subpopulations which react with the peptides or/and the complexes of peptides and MHC molecules as well as diagnostic and therapeutic uses of these compounds.

The elucidation of the molecular interrelations in the development of autoimmune diseases such as rheumatoid arthritis and juvenile diabetes (IDDM) has rapidly advanced in recent years and has meanwhile revealed concrete applications for early diagnosis and for a causal therapy of these diseases.

It is now certain that, in addition to a genetic disposition, environmental factors also play a role in the development of these diseases. For example in the case of IDDM, at the genetic risk factor level, only a few alleles of the MHC class II antigens are closely associated with this disease. Thus it is possible to define a risk group for IDDM by an analysis of these alleles (cf. e.g. Thomson et al., Am. J. Hum. Genet. 43 (1988), 799-816 or Todd et al., Nature 329 (1987), 599-604).

The environmental factors involved in the development of IDDM are probably exogenic peptide sequences that act as an immunogen. Viral antigens have been discussed among others in this connection which have partial homologies to endogenous structures. Under particular

circumstances, in particular in the postnatal phase, antigens taken up through the food such as bovine serum albumin can induce an immune response which can trigger an autoaggressive process due to homologies to endogenous structures.

The progressive destruction of the pancreatic β cells by cytotoxic lymphocytes is typical for the disease course in IDDM. This process starts a long time before there is a manifest disorder of glucose metabolism. Over 90 % of the β cells are already destroyed when there is a detectable manifestation of diabetes. The early detection of these autoaggressive T cells in persons at risk would therefore be extremely important in order to provide a causal therapy for the affected individuals.

Nowadays it has been established that the destruction of endogenous tissues in autoimmune diseases progresses very slowly at first. In the initial stage of this process the autoaggressive T cells probably only recognize one or a few auto-antigens. The work of Kaufman et al (Nature 368 (1993), 69-72) and Tisch et al (Nature 368 (1993), 72-78) on an animal model (NOD mouse) of type I diabetes have shown that in the case of spontaneously occurring diabetes in this mouse strain the initial T cell-mediated autoimmune reaction is directed towards glutamic acid decarboxylase. In this case only 1 to 2 epitopes at the C terminus of glutamic acid decarboxylase (GAD) are recognized initially in the NOD mouse. At this time it is not yet possible - as set forth above - to determine any changes in glucose metabolism whereas a perinsulitis is already detectable. Only in the further course of the disease does the spectrum of GAD peptides recognized by the autoaggressive T cells expand. After the diabetes

becomes manifest, it is also possible to detect pre-activated T cells against other islet cell antigens e.g. peripherin, heat shock protein HSP 65 and carboxypeptidase H.

There is evidence that in the case of humans the immune response to GAD is also causally linked to the development of type I diabetes. Thus for example it is possible to detect auto-antibodies against GAD in over 80 % of pre-diabetics, although however, the aetiological role of these auto-antibodies is judged to be of minor importance. On the contrary it is assumed that there is a progressive destruction of pancreatic β cells by T lymphocytes in type I diabetes. These T lymphocytes directed towards GAD have already been detected by several research groups (Harrison et al., J. Clin. Invest. 89 (1992), 1161; Honeyman et al., J. Exp. Med. 177 (1993), 535). The auto-antibodies found by these groups reacted with a peptide fragment composed of the amino acids 208 to 404 of the GAD 67 kd molecule.

Polypeptides from the human GAD 65 kd molecule which react autoimmunely are disclosed in EP-A-0 519 469. These polypeptides have the amino acid sequence:

X-P-E-V-K-(T or E)-K-Z,

in which X is an optional sequence selected from 1 to 10 amino acids and Z is an optional sequence selected from 1 to 8 amino acids.

An object of the present invention was to provide new auto-reactive peptides which react with T cells from type I diabetics, in particular with T cells from

recently discovered type I diabetics and thus define early auto-epitopes.

This object is achieved by peptides, peptide derivatives or molecules which bind analogously which are suitable for the detection, isolation, proliferation, anergization or/and elimination of auto-reactive T cells. A subject matter of the invention is therefore a peptide or peptide derivative comprising:

- (a) the amino acid sequence (I)
G-M-A-A-L-P-R-L-I-A-F-T-S-E-H-S-H-F-S-L-K-K-G-A-A,
- (b) the amino acid sequence (II)
E-R-G-K-M-I-P-S-D-L-E-R-R-I-L-E-A-K-Q-K,
- (c) one of the amino acid sequences shown in Fig. 1 or 2,
- (d) partial regions of the amino acid sequences shown in (a), (b) or/and (c) with a length of at least 6 amino acids or/and
- (e) amino acid sequences which have a specificity or/and affinity of binding to MHC molecules which is essentially equivalent to that of the amino acid sequences shown in (a), (b), (c) or/and (d).

A peptide or peptide derivative according to the invention preferably comprises

- (a) the amino acid sequence (I),
- (b) the amino acid sequence (II),
- (c) partial regions of the amino acid sequences (I) or/and (II) or/and

- (d) amino acid sequences with an essentially equivalent specificity or/and affinity of binding to MHC molecules as the amino acid sequences from (a), (b) or/and (c).

A peptide or peptide derivative according to the invention preferably comprises the partial region

L-P-R-L-I-A-F-T-S-E-H-S-H-F

of the amino acid sequence (I) or a sequence derived therefrom in which the N-terminal sequence L-P and the C-terminal sequence H-F are conserved.

The amino acid sequence (I) corresponds to the amino acid residues 266 - 290 of the human GAD 65 and the amino acid sequence (II) corresponds to the amino acid sequence 306 - 325 of the human GAD 65. The amino acid sequences shown in Figures 1 and 2 are also partial sequences of human GAD 65.

Surprisingly it was found that peptides which correspond to the amino acid sequences 266 to 285 and 306 to 325 of the human GAD 65 specifically reacted with T cell subpopulations which were isolated from recently discovered type I diabetics. Therefore the peptides according to the invention are early auto-epitopes which can be used for a very early diagnosis of type I diabetes. Furthermore the peptides according to the invention can also be used therapeutically by inactivating the T cell population that reacts with the peptides.

Preferred examples of T cell subpopulations which react with the peptides according to the invention having amino acid sequences (I) and (II) are the T cell lines

6/7 and 6/10 or T cells with an equivalent binding specificity. The T cell lines 6/7 and 6/10 were deposited on the 10th May 1994 at the "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DSMZ), Mascheroder Weg 1b, 38124 Braunschweig, Germany under the numbers DSM ACC2172 (6/7) and DSM ACC2173 (6/10) according to the regulations of the Budapest Treaty.

The amino acid sequences (I) and (II) are partial regions of the 65 kd isoform of human glutamic acid decarboxylase (GAD), the complete amino acid sequence of which has been described by Bu et al (Proc. Natl. Acad. Sci. USA 89 (1992), 2115 ff). The amino acid sequences (I) and (II) were found by setting up T cell lines from the peripheral blood of type I diabetics and subsequent in vitro stimulation with GAD from pig brain and testing these T cell lines in a proliferation assay with synthetic peptide sequences which were derived from the human GAD sequence.

The peptides according to the invention can be produced by well-known synthesis processes using chemical methods or they can be produced by genetic engineering by cloning and expression of a DNA sequence coding for these peptides in a suitable host cell, in particular E. coli.

In addition the present invention also includes peptides with partial regions of the specifically stated amino acid sequences (I) or (II) or of the amino acid sequences shown in Figures 1 and 2 which have a length of at least 6 amino acids, preferably at least 8 amino acids, particularly preferably of at least 10 amino acids and most preferably of at least 15 amino acids.

The minimum length of a peptide according to the invention is determined by its capability to recognize a MHC molecule, to bind specifically to it and to react with the corresponding T cell receptor.

The maximum length of the sections in a peptide according to the invention that are derived from GAD and bind MHC is preferably 100 amino acids, particularly preferably 50 amino acids and most preferably 25 amino acids.

In addition to peptides having the amino acid sequences (I) and (II) or partial regions thereof, the invention also concerns peptides with amino acid sequences which exhibit an essentially equivalent specificity or/and affinity of binding to MHC molecules as the aforementioned sequences and which are preferably derived by substitution, deletion or insertion of individual amino acid residues or short sections of amino acid residues from the amino acid sequences (I) or (II) or alienated substances which bind analogously.

The present invention also particularly concerns peptide variants whose sequence does not completely correspond to the above-mentioned amino acid sequences but which have the same or closely related "anchor positions". In this connection the terms "anchor position" means an amino acid residue essential for binding to a MHC molecule in particular to a MHC molecule of classes DR3, DR4 or DQ. The anchor positions for the DRB10401 binding motive are for example given in Hammer et al., Cell 74 (1993), 197-203. Such anchor positions are conserved in peptides according to the invention or optionally replaced by amino acid residues with chemically very

closely related side-chains (e.g. alanine by valine, leucine by isoleucine and vica versa). The anchor positions in the peptides according to the invention can be determined in a simple manner by testing variants of the above-mentioned specific peptides for their binding capability to MHC molecules. Peptides according to the invention are characterized in that they exhibit an essentially equivalent specificity or/and affinity of binding to MHC molecules as the aforementioned peptides. The peptides derived from peptides having the amino acid sequences (I) or (II) or from the amino acid sequences shown in Figures 1 and 2 preferably have a sequence homology of at least 30 %, particularly preferably of at least 50 % and most preferably of at least 60 % to the parent peptides or partial sequences thereof.

Examples of variants of the specifically stated peptides are the corresponding homologous peptide sections from human GAD 67, the complete amino acid sequence of which has also been described by Bu et al., supra.

The term "essentially equivalent specificity or/and affinity of binding to MHC molecules" also includes an improved binding specificity or/and affinity compared to the amino acid sequences (I), (II) or the amino acid sequences shown in Figures 1 and 2 which is found particularly in the case of truncated peptides which have a length of preferably 8 to 15 amino acids.

Moreover the present invention also includes peptide derivatives. This term includes peptides in which one or several amino acids have been derivatized by a chemical reaction. Examples of peptide derivatives according to the invention are in particular those molecules in which

the backbone or/and reactive amino acid side groups e.g. free amino groups, free carboxyl groups or/and free hydroxyl groups have been derivatized. Specific examples of derivatives of amino groups are sulfonic acid or carboxylic acid amides, thiourethane derivatives and ammonium salts e.g. hydrochlorides. Examples of carboxyl group derivatives are salts, esters and amides. Examples for hydroxyl group derivatives are O-acyl or O-alkyl derivatives. Furthermore the term peptide derivative according to the present invention also includes those peptides in which one or several amino acids are replaced by naturally occurring or non-naturally occurring amino acid homologues of the 20 "standard" amino acids. Examples of such homologues are 4-hydroxyproline, 5-hydroxylysine, 3-methylhistidine, homoserine, ornithine, β -alanine and 4-aminobutyric acid.

Those peptides are particularly preferred which have an essentially equivalent specificity or/and affinity of binding to MHC molecules as peptides having the amino acid sequences (I) or (II) but which, in contrast to these peptides, do not cause an activation of T cells but rather the production of an anergic state in the T cells.

The present invention also covers polypeptides in which the MHC-binding peptide section is a component of a larger polypeptide unit in which the link between the MHC-binding peptide and the rest of the polypeptide unit preferably has a predetermined breaking point e.g. a protease cleavage site.

A further subject matter of the present invention is a

peptide or peptide derivative which carries a substance that generates a signal or a marker group e.g. a fluorescent marker group (e.g. rhodamine, phycoerythrin), digoxin, biotin, a radioactive group or a toxin group (e.g. ricin, cholera toxin etc.). Coupling of the peptide according to the invention to marker groups enables the peptide to be used as a diagnostic agent for in vivo or in vitro (e.g. imaging) applications or as a therapeutic agent. Furthermore the peptide according to the invention can also for example be present in a cyclised form or in an oligomeric form in which the sequences that are important for binding to the MHC molecule are separated from one another by spacer regions.

The invention also concerns peptide-mimetic substances which exhibit an essentially equivalent specificity or/and affinity of binding to MHC molecules as the aforementioned peptides or peptide derivatives. Peptide-mimetic substances or peptide-mimetics are compounds that can replace peptides with regard to their interaction with the MHC molecules and which can have an increased metabolic stability, improved bioavailability and longer duration of action compared to the native peptides. Methods for the production of peptide-mimetics are described by Giannis and Kolter, "Angew. Chem. 105 (1993), 1303-1326, Lee et al., Bull. Chem. Soc. Jpn. 66 (1993), 2006-2010 and Dorsch et al., "Kontakte" (Darmstadt) (1993) (2), 48-56. Reference is made to the disclosure of these references with regard to the production of peptide-mimetic substances according to the invention.

The present invention in addition concerns a complex which comprises at least one peptide, peptide derivative

or peptide-mimetic according to the invention and at least one MHC molecule or a peptide-binding derivative of a MHC molecule. In this complex a peptide, peptide derivative or peptide-mimetic with a binding constant of preferably at least 10^{-7} l/mol, particularly preferably in the range of 10^{-8} - 10^{-9} l/mol, is bound to a MHC molecule or peptide-binding derivative of a MHC molecule. Alternatively the peptide, peptide derivative or peptide-mimetic can also be covalently coupled to the MHC molecule e.g. by means of a photolinker or as a covalent genetic peptide-MHC fusion. Such a peptide-MHC fusion protein preferably contains a HLA-DR beta chain and an autoreactive peptide that is genetically fused thereto. The complex particularly preferably contains a MHC class II molecule or a peptide-binding derivative thereof.

The MHC class II molecule is preferably of the DR type, for example of the DR1, DR2, DR3 or DR4 type. The MHC class II molecule is particularly preferably of the subtype DR B1 101, DR B1 0301, DR B1 0401, DR B1 0402, DR B1 0404 or DR B1 1601. The MHC class II molecule of subtype DR B1 0101 or DR B1 0401 is most preferred. The T cell line 6/7 (DSM ACC2172) proliferates with the autoreactive peptide of the amino acid sequence (I) in the presence of the DR B1 allele 0401 and 0101 or/and 1601. A proliferation in the presence of the DR B1 allele 0401 is found with the autoreactive peptide having the amino acid sequence (II). The T cell line 6/10 (DSM ACC2173) proliferates with the autoreactive peptides of the amino acid sequences (I) and (II) in the presence of the DR B1 allele 0401.

The nucleotide sequences for genes coding for a MHC class II molecule of the above subtypes are published in

Corell et al. (Mol. Immunol. 28 (1991), 533-543).
Reference is hereby made to these publications.

The term "peptide-binding derivative of a MHC molecule" comprises fragments of MHC molecules which are produced by proteolytic cleavage of native MHC molecules or by recombinant DNA techniques and which have essentially preserved their peptide-binding properties. In addition this term is also understood to include fusion proteins which also contain further polypeptide components in addition to a MHC part responsible for the peptide binding.

The peptide-MHC complexes according to the invention are preferably produced by association of peptide-free MHC molecules or MHC molecule derivatives with the peptides, peptide derivatives or peptide-mimetics according to the invention. The production of peptide-free MHC molecules can for example be carried out by unfolding native MHC molecules in order to dissociate bound peptides and refolding the empty MHC molecules (see Dornmair and McConnell, Proc. Natl. Acad. Sci. USA 87 (1990), 4134-4138 and WO91/14701).

On the other hand peptide-free MHC molecules can also be obtained by recombinant production of MHC molecules or derivatives thereof. Examples of this are the expression of MHC class II molecules in fibroblasts (Germain and Malissen, Ann. Rev. Immunol. 4 (1990), 281-315) as well as the expression of soluble MHC class II molecule derivatives without a membrane anchor in CHO cells (Wettstein et al., J. Exp. Med. 174 (1991), 219-228, Buelow et al., Eur. J. Immunol. 23 (1990), 69-76) and by means of the baculovirus expression system in insect

cells (Stern and Wiley, Cell 68 (1992), 465-477; Scheirle et al., J. Immunol. 149 (1992), 1994-1999). MHC class I molecules have also been expressed in CHO cells (Fahnestock et al., Science 258 (1992), 1658-1662), in insect cells (Jackson et al., Proc. Natl. Acad. Sci. USA 89 (1992), 12117-12120; Matsamura et al., J. Biol. Chem. 267 (1992), 23589-23595) and in fibroblasts (Mage et al., Proc. Natl. Acad. Sci. USA 89 (1992), 10658-10661).

In addition it is also known that peptide-free MHC molecules can be expressed in E. coli (Parker et al., Mol. Immunol. 29 (1992), 371-378; Zhang et al., Proc. Natl. Acad. Sci. USA 89 (1992), 8403-8407; Garboczi et al., Proc. Natl. Acad. Sci. USA 89 (1992), 3429-3433; Altman et al., Proc. Natl. Acad. Sci. USA 90 (1993), 10330-10334). Reference is made for the present invention to the techniques for the recombinant expression of MHC molecules or MHC molecule derivatives described in these publications.

The MHC component of the complex according to the invention is preferably a recombinant MHC molecule or a peptide-binding derivative thereof and particularly preferably a soluble MHC molecule derivative in which the membrane anchor is partially or completely deleted.

In order to identify MHC molecules which present the autoreactive peptide according to the invention, the antigen-presenting cells of a donor are incubated with the peptide according to the invention in a labelled form and bound peptides are preferably firstly dissociated by denaturation of native MHC molecules. Subsequently the labelled MHC-peptide complexes can be immunoprecipitated with subtype specific antibodies

which are directed towards framework-specific determinants of the MHC molecules and identified by the presence of the labelled peptides.

Alternatively EBV (Epstein-Barr virus) transformed B cells of the donor can be used as the antigen-presenting cells.

The production of the complexes according to the invention from a recombinant MHC molecule derivative can for example be carried out in such a way that DNA fragments for the soluble parts of the α and β chains of a MHC molecule, e.g. a MHC DR3, DR4 or DQ molecule, are isolated by PCR using cDNA from an EBV transformed B cell line of the donor as the template which expresses the corresponding MHC molecule. In this step it is preferable to introduce a purification aid, e.g. an oligohistidine segment (e.g. a hexahistidine segment), at the C terminus of the α and β chain by appropriate selection of the PCR primer. The PCR products can be subsequently subcloned in *E. coli* and expressed as inclusion bodies. The inclusion bodies can be solubilized by known methods (cf. references on the expression of MHC molecules in *E. coli*, supra) and the MHC proteins can be purified by means of metal chelate affinity chromatography. Subsequently the α and β subunits are renatured in the presence of the peptide.

The peptide-MHC complex according to the invention can also carry a marker group as described above in which case the marker group can be bound by known methods to the peptide component as well as to the MHC component of the complex.

A further subject matter of the present invention is an oligomerized peptide-MHC complex which contains at least two MHC molecules or MHC molecule derivatives that are associated by means of covalent or non-covalent interactions. An advantage of such an oligomerized peptide-MHC molecule complex compared to known monomeric complexes (with regard to the MHC molecule) is that it has a higher affinity and thus an improved diagnostic or/and therapeutic efficacy.

In one embodiment of the present invention such an oligomerized complex can be produced according to known methods by covalent cross-linking of monomeric peptide-MHC complexes via chemical coupling reagents e.g. N-succinimidyl-3(2-pyridylthio)propionate, 3-maleimido-benzoyl-N-hydroxysuccinimide ester, maleimidohexanoyl-N-hydroxysuccinimide ester, bis(maleimidomethyl)ether, disuccinimidyl suberate, glutardialdehyde etc.. Optionally it is also possible to modify individual amino acids of the peptide component or the MHC component in such a way that special coupling reagents preferably attack at this position. Thus coupling by SH linkers or via amino groups can be achieved by introducing additional cysteine or lysine residues by recombinant means in the case of the protein component or by chemical synthesis in the case of the peptide component.

In a further embodiment of the present invention the oligomerized peptide-MHC complex can be produced in such a way that the peptide component binding to the MHC molecule is used as an oligomer i.e. as a peptide molecule that contains at least 2 MHC-binding regions in which the sequences that are important for binding to the MHC molecule are separated from one another by

spacer regions. These spacer regions are usually comprised of 10 - 15 amino acids. Small hydrophilic amino acids such as glycine, alanine, serine, proline or combinations thereof are used. When peptide-free MHC molecules are renatured in the presence of these peptide oligomers the oligomerized complex according to the invention is formed which contains MHC molecules cross-linked by the oligomerized peptide components via non-covalent interactions.

In addition oligomerized peptide-MHC complexes can be produced by modifying recombinantly produced MHC molecules. Thus during the construction of vectors for the expression of recombinant α or β chains of MHC class II molecules it is possible to clone in a gene segment, preferably at the C-terminus, which codes for an epitope that is recognized by an antibody. This antibody can be of the IgG type but preferably of the IgM type. The renatured monomeric peptide-MHC complexes are then incubated with an antibody that recognizes the introduced epitope so that non-covalently cross-linked immune complexes composed of several antibodies and several peptide-MHC complexes are produced. The introduction of DNA segments that code for an epitope into the DNA fragments coding for the α or β chain of the MHC molecule can be achieved by means of well-known molecular biological techniques e.g. by insertion into restriction sites or by site-directed mutagenesis.

The oligomerized peptide-MHC complex according to the invention preferably contains a peptide which comprises the amino acid sequences (I), (II), the amino acid sequences shown in Figures 1 and 2, partial regions thereof or/and amino acid sequences derived therefrom, or a peptide derivative or peptide-mimetic thereof. The

oligomerized complex can be preferably used as a diagnostic or therapeutic reagent in type I diabetes.

The invention therefore also concerns a pharmaceutical composition containing a peptide, peptide derivative, peptide-mimetic or/and a peptide-MHC complex as the active component, if desired, in combination with common pharmaceutical additives. The composition can in addition contain an accessory stimulating component e.g. cytokines such as IL-2 and IL-4 or/and the surface antigen B7 (Wyss-Coray et al., Eur. J. Immunol. 23 (1993), 2175-2180; Freeman et al., Science 262 (1993), 909-911) which can bind to the surface molecule CD-28 on a T cell. The presence of the accessory stimulating component can improve or/and modify the therapeutic effect of the composition.

Furthermore the present invention concerns the use of a pharmaceutical composition which contains a peptide, peptide derivative, peptide-mimetic or/and a peptide-MHC complex for the production of an agent for the diagnosis of diseases or of a predisposition to diseases that influence the immune system or for the diagnosis of tumour diseases or of a predisposition to tumour diseases, in particular for the diagnosis of autoimmune diseases or of a predisposition to autoimmune diseases, e.g. diabetes type I or type II, preferably diabetes type I.

Analogous diagnostic applications are, however, also possible in the case of other autoimmune diseases. Examples of such autoimmune diseases are multiple sclerosis where reactive T cells against the myelin basic protein or the proteolipid protein can be

determined, rheumatoid arthritis where reactive T cells against collagen type II, cytokeratins and Hsp 65 can be determined, Basedow disease where reactive T cells against thyroid peroxidase can be determined.

In general a diagnostic application is possible for all diseases that influence the immune system such as e.g. also in the case of arteriosclerosis. In this case the disease has been shown to be associated with an immune response against the heat shock protein Hsp 65 (Xu et al., Lancet 341, 8840 (1993), 255-259).

A further application is the diagnostic detection of T cells which react to tumour antigens. Examples of this are T cells against a melanoma-associated antigen MAGE 1 which has been isolated from melanoma patients (van der Bruggen et al., Science 254 (1991), 1643-1647). The oligomerized complexes according to the invention can be used to detect these T cells at a stage in which the tumour is not yet detectable by conventional methods due to an inadequate cell mass. Moreover the detection of specifically reacting T cells can also be used to monitor an anti-tumour vaccination.

The present invention therefore also concerns a method for the determination of a specific T cell subpopulation which is characterized in that a sample containing T cells, which is preferably derived from a body fluid e.g. whole blood, is brought into contact with a peptide, peptide derivative, peptide-mimetic according to the invention or/and a complex according to the invention and the reaction of T cells with the peptide or complex is determined. A specific reaction of T cells with the complex or the peptide can for example be

detected by an increased T cell proliferation which can for example be measured by the incorporation of radioactivity. On the other hand the reaction of T cells can also be determined directly by the use of a labelled peptide or complex. In this embodiment the peptide or the complex are preferably used with a fluorescent marker group coupled thereto. The evaluation can for example be carried out by FACS analysis in which the T cells are brought into contact with a first fluorescent marker that is coupled to a T cell-specific antibody and then with the peptide-MHC complex that is coupled to a second fluorescent marker and the presence of double labelled cells is determined by fluorographic analysis. In this way a T cell subpopulation is determined which is characterized by its reactivity with a peptide or peptide derivative according to the invention or/and with a peptide-MHC complex according to the invention. Due to the low concentration of the specific T cell population in blood, a selection for pre-activated T cells is preferably carried out as the first step of the method e.g. a selective concentration of IL-2 receptor-positive T cells by incubation with IL-2 or/and by incubation with IL-2 receptor antibodies and subsequent separation of the antibody-binding cells by using immunomagnetic methods for example. On the other hand the selection for pre-activated cells can be carried out after contact of the T cells with the peptide or the complex.

In a modification of the method the ratio of the pre-activated autoreactive T cells, i.e. T cells with the IL-2 receptor as the surface marker, to non-activated autoreactive T cells, i.e. T cells without the IL-2 receptor, can also be determined.

This method can be used in particular to diagnose type I diabetes and also for other diseases that influence the immune system or for the diagnosis of a predisposition to such diseases.

The present invention in addition concerns the use of a pharmaceutical composition which contains a peptide, peptide derivative, peptide-mimetic or/and a peptide-MHC complex according to the invention for the production of an agent for the therapy or prevention of diseases that influence the immune system. For the therapeutic application of the peptides according to the invention and the peptide-MHC complexes according to the invention, it is possible for example to use peptides or peptide-MHC complexes coupled to toxins, on the other hand it is also possible to use peptides alone or as components of the complex which, although enabling binding to the T cell receptor, do not cause activation of T cells i.e. have an anergizing effect.

The therapeutic action of such anergizing peptide analogues is due to the fact that the T cell receptor (TCR) must interact with a peptide which is presented by a MHC antigen of class I or class II in order to activate the T cells. In this process amino acids at the anchor positions of the peptide are particularly responsible for binding to the MHC molecule whereas other amino acids in the peptide contribute to the interaction with the TCR and thus cause a T cell stimulation. Thus it is possible to produce peptide analogues by amino acid substitutions in the peptides which still bind to the MHC molecule due to the presence of the anchor positions but on the other hand only cause a partial or no T cell activation (cf. e.g. Sloan-Lancaster et al., Nature 363 (1993), 156-159). Such

peptide analogues can for example have the effect that the expression of particular surface molecules is set to a higher level (e.g. IL-2 receptor, LFA-1) but that no proliferation or cytokine expression takes place. T cells which interact with such a peptide analogue are transformed into a so-called anergic state i.e. they can no longer proliferate even after a subsequent regular stimulation with an immunogenic peptide. This anergic state lasts at least 7 days and can therefore be utilized therapeutically in the treatment of an autoimmune disease.

A further therapeutic aspect of the present invention is that the peptide or the complex of peptide and MHC molecule can be used as an antigen. Such an antigen can act in this case as an immunogen i.e. as an agent stimulating an immune response or as a tolerogen i.e. as an agent which causes an immune tolerance. The application as an immunogen can for example be used for vaccination against tumour antigens. Instead of the whole tumour cells hitherto used for this purpose it is possible to inject tumour-specific peptides recognized by the T cells in a complex with the corresponding MHC molecules in particular in the form of an oligomerized complex in order to produce a T cell response against the tumour. In order to increase the immune stimulation it is also possible to administer the complex in combination with additional stimulating substances. Cytokines such as IL2 or IL4 are for example suitable for this purpose which are optionally and preferably covalently linked to the peptide-MHC complex according to the invention. A further possibility is to associate the complex with accessory components for T cell activation in particular with essential surface molecules for antigen presenting cells e.g. the surface

molecule B7.

A preferred therapeutic formulation is the incorporation of MHC molecules loaded with peptides into artificial vesicles e.g. lipid vesicles which can optionally carry further membrane-bound molecules such as e.g. B7 or/and immobilized cytokines.

The present invention in addition concerns the isolation of T cell subpopulations that react with a peptide or peptide-MHC complex according to the invention. In such a method a sample containing T cells which is for example derived from a body fluid that has previously been collected from a patient, is contacted with a peptide according to the invention or with a peptide-MHC complex according to the invention, the T cells that react with the peptide or complex are identified and if desired they are separated from other T cells. Also in this case it is possible to select for pre-activated T cells, i.e. T cells with the IL-2 receptor, before or/and after contact of the T cells with the peptide or the complex.

In such a method the peptide or peptide-MHC complex can be used in an immobilized form on a carrier which simplifies the separation of the positively reacting T cell population from other T cells. T cell lines can be set up by restimulation from the T cell subpopulations isolated in this manner. These autoreactive T cell lines can then be used to immunize patients.

A specific immunotherapy of type I diabetes comprises firstly isolating specific T cell lines against an autoantigen e.g. GAD 65 from IDDM patients. Then the

fine specificity of the T cell lines is determined i.e. the autoreactive peptides are identified. Those T cell lines are selected for the later vaccination of the patients which recognize a predominant peptide i.e. a peptide to which several of the isolated T cell lines react. These are in particular T cell lines which recognize a peptide with the amino acid sequences (I) or (II).

If there is no clearly predominant peptide in a patient, several T cell lines have to be mixed for the later inoculation. The selected T cell clones are stimulated again before inoculation with antigen-presenting cells and with the appropriate peptides in order to ensure a good expression of activation molecules and in particular of the T cell receptors. Then the T cell lines are inactivated e.g. by heat treatment or/and radioactive irradiation preferably with a dose in the range of 4000 - 10000 rad, particularly preferably ca. 8000 rad and injected subcutaneously into the patient from which they were obtained using a cell number of preferably 10^7 to 5×10^7 . Usually at least three injections are spread over a time period of 6 to 12 months.

Subsequently the T cell response of the patient to the inoculate can be tested. For this the peripheral blood lymphocytes (PBLs) of the patient are isolated e.g. by means of Ficoll density gradient centrifugation and the proliferation caused by the inoculate is tested in a standard proliferation test. After a successful immunization it should be possible to detect a substantial proliferation of the patient's PBLs in response to the inoculate. A further control of the success of immunization is to determine the frequencies

After the surface structures on the T cells of the inoculate that are recognized by the regulatory T cells have been narrowed down further, it is also possible to immunize with partial structures of the regulatory T cells e.g. with segments of the T cell receptor.

In diagnostic and therapeutic methods for identifying or activating/inhibiting specific T cell subpopulations, an anti-idiotypic antibody which simulates the action of the MHC-peptide complex can also be used instead of the peptides or peptide-MHC molecules according to the invention. Such antibodies can be easily obtained by using a specific T cell subpopulation against a particular peptide as an immunogen for producing an antibody (e.g. in a mouse) or by firstly producing a first antibody against the MHC-peptide complex and then producing an anti-idiotypic antibody against the first antibody.

Therefore a subject matter of the present invention is also an antibody (first antibody) against a peptide or

peptide derivative according to the invention or against a complex according to the invention which is obtainable by immunizing with the peptide, peptide derivative or complex and isolating an antibody produced by the immunization, preferably a monoclonal antibody produced by the method of Köhler and Milstein or further developments thereof.

Finally the invention also concerns an anti-idiotypic antibody against the first antibody obtainable by immunizing with the first antibody which is directed towards the peptide or peptide derivative or complex and isolating an anti-idiotypic antibody produced by the immunization.

Yet a further subject matter of the present invention is a T cell which reacts with an autoreactive peptide, peptide derivative or peptide-mimetic according to the invention or a complex of the peptide and MHC molecule. Preferred examples are T cells that are derived from the T cell lines 6/7 (DSM ACC2172) or 6/10 (DSM ACC2173) or which have an equivalent T cell receptor binding specificity i.e. recognize a peptide presented by a MHC molecule or peptide derivative having the amino acid sequences (I) or/and (II) or/and partial regions of these amino acid sequences.

It is intended to further elucidate the invention by the following examples in conjunction with Figures 1 to 4.

Fig. 1 shows autoreactive amino acid sequences according to the invention

Fig. 2 shows further autoreactive amino acid sequences

according to the invention

Fig. 3 shows the result of a proliferation assay of the T cell line 6/7 with peptide pools

Fig. 4 shows the result of a proliferation assay of the T cell line 6/10 with peptide pools

Fig. 5 shows the result of a proliferation assay with the T cell line 6/7 with individual peptides from pool 7 and 11

Fig. 6 shows the result of a proliferation assay with the T cell line 6/10 with individual peptides from pool 7 and 11

EXAMPLE 1

Setting up GAD-specific T cell lines

1. Primary stimulation

Peripheral blood lymphocytes (PBLs) are isolated from EDTA blood of type I diabetics by Ficoll density gradient centrifugation. The cells are washed twice in RPMI medium and then taken up in a culture medium composed of RPMI 1640, 5 % human serum, 2 mM glutamine and 100 U/ml penicillin and 100 µg/ml streptomycin. 100 µl cell suspension, corresponding to 100 000 cells, are sown into each well of a 96 well round-bottom plate. Afterwards porcine GAD (SW-GAD) is added at a final concentration of 2.5 µg/ml. The cells are incubated for 3 - 4 days in an incubator at 37°C/7 % CO₂. After this time period, 100 µl IL-2 (30 U/ml) is added. After a further 3 - 4 days, 100 µl is aspirated from all culture

mixtures and again 100 μ l IL-2 (30 U/ml) is added. This is repeated every 3 - 4 days.

2. Restimulation

The first restimulation is carried out on the 14th day after the beginning of the primary stimulation. For this, in comparison to the primary stimulation, double the number of autologous PBLs is isolated by means of Ficoll and adjusted in culture medium to a cell concentration of 2×10^6 /ml. One half of these stimulator cells is incubated for 2 hours/37°C/7 % CO₂ with the antigen SW-GAD (final concentration 5 μ g/ml) (antigen pulse). The other half is incubated under the same conditions without antigen and only with culture medium. Subsequently all stimulator cells are irradiated with 4000 rad. The stimulator cells are then distributed (100 000 cells/well) in 96 well round-bottom plates in such a way that a well with stimulator cells containing antigen is always adjacent to a well with stimulator cells without antigen.

Subsequently the T cells are prepared from the primary stimulation preparations. For this the supernatants from the primary stimulation preparations are aspirated and the cells in the plates are washed twice with 100 μ l washing medium each time (Dulbecco's Modified Eagle Medium = DMEM). Between these the cells are centrifuged in the plates at 400 g. Subsequently the cells are taken up in 100 μ l culture medium in each case and 50 μ l thereof is distributed in each case into two adjacent wells of the restimulation plate. In this manner the T cells are incubated in one well with antigen and in the adjacent well without antigen it is possible to monitor the antigen specificity of the restimulation.

From the 2nd or 3rd day after the start of the restimulation it is possible to assess the proliferation by microscopy. In this process only those microculture pairs are regarded as being relevant in which proliferation only occurs in the well in which antigen is present. From the 4th day 100 μ l IL-2 (30 U/ml) is in turn added to each culture well. Up to the 14th day ca. 50 % of the culture medium is exchanged for IL-2 (30 U/ml) every 3 - 4 days.

When the growth is good the cultures are divided among several 96 well plates. In the later restimulation it is also possible to distribute them into larger wells. A renewed restimulation is carried out by the aforementioned method every two weeks. From the 3rd restimulation onwards the specificity of the microcultures is determined in a proliferation test.

3. Proliferation test with SW-GAD

All tests are carried out in at least duplicate preparations.

a) Stimulator cells:

Autologous PBLs or PBLs of a normal donor which are identical with regard to the HLA class II antigens are used as stimulator cells. The PBLs are pre-incubated with antigen as described in paragraph 2, irradiated with 4000 rad and distributed in 96 well plates (100 000 cells/well).

b) T cells

The T cells which were used are derived always from the final stage of a restimulation period. They are washed three times free of antigen and IL-2 using

DMEM and divided into 6000 cells/96 wells. The T cell lines 6/7 and 6/10 isolated in this manner were deposited at the DSM according to the regulations of the Budapest Treaty under the numbers DSM ACC2172 and DSM ACC2173.

In addition to the incubations with SW-GAD, controls are incubated without GAD.

After 3 - 4 days at 37°C/7 % CO₂, 1 µCi ³H-thymidine is added and incubated for a further 16 - 20 hours. Afterwards the cells are transferred onto a glass fibre filter by means of a cell harvester instrument and the incorporated radioactivity is determined in a β counter.

Table 1 shows a typical result of a proliferation test with SW-GAD

Table 1:

Results of a proliferation test of T cell lines 6/7 and 6/10 with SW-GAD

Cell line	Control without antigen	cpm SW-GAD
6/7	129	9373
6/10	117	5222

4. Proliferation test with peptides that are derived from the H-GAD sequence

T cell lines that had been expanded by means of at least 4 restimulation cycles and which reacted with SW-GAD in the proliferation test were additionally tested with overlapping peptides of H-GAD. The object of these experiments is to define the epitopes of H-GAD that are recognized by the T cells. For this, overlapping 20 mer peptides of H-GAD are firstly synthesized (overlapping region 10 amino acids, total of 59 different peptides).

In each case 4-5 of these peptides are combined to form a pool and added to the stimulator cells at a final concentration of 18 $\mu\text{g/ml}$ in each case (the stimulator cells are prepared as described in section 3a). The further treatment of these stimulator cells is carried out as described in section 3a.

Subsequently 6000-20,000 T cells are added per microculture well. The further procedure is analogous to that described in section 3b.

Figures 3 and 4 show the results of a proliferation assay of the T cell lines 6/7 and 6/10 using peptide pools of the human GAD 65 kd. Both T cell lines proliferate strongly with peptides from pool 11. A smaller but significant proliferation is also observed with pool 7.

Figures 5 and 6 show the results of the proliferation assay of the T cell lines 6/7 and 6/10 with 10 $\mu\text{g/ml}$ individual peptides from pools 7 and 11. Both cell lines show a significant proliferation with peptide 5G1

(corresponding to amino acids 266-285 of human GAD 65) and a smaller but significant proliferation with peptide 5F3 (corresponding to amino acids 306-325 of human GAD 65).

EXAMPLE 2

Method for the isolation and determination of an antigen-specific T cell subpopulation

Since antigen-specific T lymphocytes and particularly those directed towards autoantigens occur in peripheral blood in a very low number (expected frequency 10^{-5} - 10^{-6}) the obvious thing is to firstly concentrate the in vivo pre-activated T cells by means of a selection step. This can be achieved by two methods:

1. Expansion of in vivo pre-activated T cells by incubation with IL-2

For this PBLs are isolated by means of Ficoll density gradient centrifugation and adjusted in cell culture medium containing IL-2 (RPMI 1640/5 % human serum/ 30 U/ml IL-2) to 2×10^6 cells/ml. 200 μ l aliquots of the cells are distributed among 48 well plates and incubated for 7 days. After 4 days IL-2 is additionally added once again. Since pre-activated T cells express the high affinity IL-2 receptor, the in vivo pre-activated T cells proliferate selectively during this stimulation period and accumulate in the primary culture. After the conclusion of the stimulation period, the cells are washed in the individual wells, counted and used in a proliferation test.

2. Concentration of in vivo pre-activated T cells by immunomagnetic separation

For this the Ficoll-isolated PBLs are incubated with monoclonal antibodies against the high affinity IL-2 receptor (7×10^6 PBLs/ml; $10 \mu\text{g/ml}$ anti IL-2 receptor antibody (Boehringer Mannheim); 30 min at 4°C). Subsequently the cell suspension is washed twice with ice-cold RPMI/10 % human serum (HS) (400 g/10 min) and then the suspension is adjusted to a cell density of $1 - 3 \times 10^7/\text{ml}$. Dynabeads M-280 from the Dynal company that are coupled to sheep anti-mouse antibodies are added to this (ratio of Dynabeads to target cells ca. 10 - 15). The suspension is moved very slowly at 4°C on a roller. Afterwards the suspension is diluted ten-fold with RPMI/10 % HS and placed for 1 - 2 minutes in the previously cooled magnetic particle concentrator (MPC). After the rosetted T cells carrying the IL-2 receptor have been immobilized by the magnet, the supernatant is aspirated, the incubation vessel is removed from the MPC and the remaining cells are resuspended in RPMI/10 % HS. The separation of the target cells is carried out once again in MPC. This washing step is repeated once again. Subsequently the separated cells are resuspended in culture medium and the cell number is adjusted to $1 \times 10^7/\text{ml}$. The magnetobeads are removed by known methods by means of detacher antibodies.

The pre-activated cells concentrated by means of the processes according to 2.1 or 2.2 are subsequently tested for reactivity against the auto-antigen peptides or against a peptide/MHC complex.

There are also several methods for this:

3. Proliferation test with irradiated stimulator cells and peptides as antigens

Firstly autologous stimulator cells are prepared from Ficoll-isolated PBLs. The stimulator cells are adjusted to a concentration of 10^6 cells/ml in cell culture medium and incubated with the peptides (final concentration $10 \mu\text{g/ml}$) for 2 hours/ $37^\circ\text{C}/7\% \text{ CO}_2$. Afterwards the stimulator cells are irradiated with 4000 rad and subsequently distributed in a 96-well round-bottom plate using a cell number of 100 000 cells/well.

100 000 of the in vivo pre-activated T cells obtained from 2.1 or 2.2 are added to this in each case and incubated for 4 days/ $37^\circ\text{C}/7\% \text{ CO}_2$. Then half of the preparation volume is exchanged for IL-2 (30 U/ml). This is repeated once again after a further 4 days.

On the 12th day after the start of the microculture, the actual proliferation test is carried out. For this the microcultures are firstly washed twice with DMEM in order to remove antigen and IL-2. Each culture is divided into 4 aliquots and incubated ($37^\circ\text{C}/7\% \text{ CO}_2$) for 3 days in duplicates in the presence of 100 000 autologous irradiated PBLs, in each case with or without a peptide pulse. After this period ^3H -thymidine is added and the incorporated radioactivity is determined after a further 16 - 20 hours.

4. Direct detection of autoantigen-reactive T cells by labelling by means of an oligomerized peptide/HLA complex

The in vivo pre-activated T cells concentrated according to method 2.2 are used for this. The cells are adjusted in RPMI/10 % HS to a concentration of $10^6/\text{ml}$ and

incubated at 4°C for 30 minutes with the oligomerized HLA-peptide complex provided with a fluorescent label. Subsequently the cells are washed twice with ice-cold cell culture medium. The analysis of the fluorescent-labelled cell population is carried out in a flow cytometer.

EXAMPLE 3

Identification of MHC molecules that present a defined autoreactive peptide

The peptides are firstly labelled with ^{125}I for this e.g. according to the method of Bolton and Hunter (Bolton, A.E. and Hunter, W.M., Biochem. J. 133 (1993), 529-531). Then $2 - 5 \times 10^6$ PBLs of the donor having the MHC-type to be examined are incubated for 4 hours at 37°C in cell culture medium containing ^{125}I -labelled peptides ($2 - 10 \mu\text{M}$). After washing the cells, these are lysed in a lysis buffer composed of 0.5 % NP 40; 0.5 % Mega9; 150 mM NaCl; 5 mM EDTA; 50 mM Tris pH 7.5; 2 mM phenylmethylsulfonyl fluoride). The MHC molecules are immunoprecipitated from the mixture by framework-specific monoclonal antibodies (e.g. with the monoclonal antibody L243 (ATCC HB 55) in the case of HLA-DR) bound to protein A-Sepharose and the radioactivity bound to the protein A-Sepharose is determined in a gamma counter.

EXAMPLE 4

Determination of the subtype of MHC molecules that present autoreactive peptides to the T cell lines 6/7 (DSM ACC2172) and 6/10 (DSM ACC2173)

The experimental procedure is analogous to example 1.3. However, autologous PBLs were not used as antigen-presenting cells, but instead PBLs from heterologous donors which do not completely correspond to the MHC molecules of the donor from which the T cell lines were also developed but only with regard to defined MHC alleles. The proliferation tests were carried out using the autoantigenic peptides 5G1 (corresponding to amino acids 266-285 of human GAD65) and 5F3 (corresponding to amino acids 306-325 of human GAD65).

Table 3 shows the result of such a test mixture. The T cell lines 6/7 and 6/10 proliferate with both peptides in the presence of the DR B1-allele 0401. Variation of the DQ A1 or DQ B1 allele does not have an influence on the stimulatibility of the T cell lines. The T cell line 6/7 recognizes the peptide 5G1 additionally in association with the alleles DR B1 0101 or/and 1601.

663440614304163

T cell proliferation after stimulation with the peptides 5G1 and 5F3 using PBLs with various haplotypes as antigen-presenting cells

Table 3

Donor	Haplotype of APC DR B1* DQ A1* DQ B1*			Identicalness of the alleles with the alleles of the donor of the TCL	TCL 6/7 cpm +peptide SI		TCL 6/10 cpm +peptide SI	
A.K.	0301	0501	0201	DR: 2 alleles ident. DQ: 4 alleles ident.	5G1	55.0	5G1	6.0
	0401	0301	0302		5F3	3.8	5F3	3.8
G.H.	0301	0501	0201	DR: 1 allele ident. 1 allele not ident. DQ: 4 allels ident.	5G1	0.9	5G1	1.5
	0404	0301	0302		5F3	0.6	5F3	1.5
G.E.	1302	0102	0604	DR: 1 allele ident. 1 allele not ident. DQ: 2 allels ident. 2 allels not ident.	5G1	67.8	5G1	22.6
	0401	0301	0302		5F3	7.0	5F3	6.5
19	0301	0501	0301	DR: 2 allels ident. DQ: 1 allele ident. 3 allels not ident.	5G1	45.7	5G1	8.5
	0401	0201	0301		5F3	3.1	5F3	2.
D.J.	0101	0101	0501	DR: 2 allels not ident. DQ: 4 allels not ident.	5G1	28.6	5G1	2.2
	1601	0102	0502		5F3	1.2	5F3	1.4

TCL = T cell line

APC = antigen-presenting cells

SI = stimulation index: cpm in the presence of peptide divided by cpm without peptide.

EXAMPLE 5

Proliferation test with variants of the peptide 5G1 using the T cell line 6/10

In order to elucidate the core structure of the stimulating peptide 5G1, proliferation tests were carried out with various variants of this peptide using the T cell line 6/10 (see Table 4).

A test with a first series of 20 mer variants is intended to ascertain whether amino acids which border on the 5G1 structure at the C- or N-terminus play a role in the recognition by the T cell line 6/10. As the stimulation indices show, a shift of the 20mer peptide in the direction of the C-terminus does not lead to an increase in the proliferation activity. A 6 amino acid shift towards the N-terminus leads to a loss in stimulation capacity.

A test with a second series of peptide variants examines the influence of a shortening at the C-terminus. This series of experiments shows that the C-terminal amino acid residues histidine (H) and phenylalanine (F) are important for the stimulation capacity.

The object of a test with a third series of peptide variants was to define the N-terminus of the minimum stimulation-active peptide. If the amino acids leucine (L) and proline (P) are removed from a 18mer with an intact C-terminus, this leads to a strong decrease of the stimulation index. The N-terminus is thus defined by L and P. If the C-terminus is shortened further by H and F this also leads to a loss of stimulation activity.

Thus in the case of the T cell line 6/10 the minimum peptide that can still stimulate is a 14mer having the sequence LPRLIAFTSEHS HF.

Table 4

Reaction of TCL 6/10 with variants of the peptide 5G1 in order to identify the minimum peptide structure which still has stimulation activity

Peptide variants of 5G1		Stimulation index
5G1	GMAALPRLIAFTSEHS HFSL	5.4
	ALPRLIAFTSEHS HFSLKKG	3.0
	RLIAFTSEHS HFSLKKGAAA	3.2
	PEVKEKGMAALPRLIAFTSE	0.6
	AALPRLIAFTSEHS HFSL	4.5
	AALPRLIAFTSEHS HF	2.9
	AALPRLIAFTSEHS	0.7
	AALPRLIAFTSE	0.6
	GMAALPRLIAFTSE	0.8
	GMAALPRLIAFT	1.0
	LPRLIAFTSEHS HFSLKK	3.2
	RLIAFTSEHS HFSL	1.4
	LPRLIAFTSEHS HF	4.6
	LPRLIAFTSEHS	0.4

CLAIMS

1. Peptide or peptide derivative comprising:
 - (a) the amino acid sequence (I)
G-M-A-A-L-P-R-L-I-A-F-T-S-E-H-S-H-F-S-L-K-K-G-A-A
 - (b) the amino acid sequence (II)
E-R-G-K-M-I-P-S-D-L-E-R-R-I-L-E-A-K-Q-K
 - (c) one of the amino acid sequences shown in Fig. 1 or 2
 - (d) partial regions of the amino acid sequences shown in (a), (b) or/and (c) having a length of at least 6 amino acids or/and
 - (e) amino acid sequences which exhibit a specificity or/and affinity of binding to MHC molecules which is essentially equivalent to that of the amino acid sequences shown in (a), (b), (c) or/and (d).
2. Peptide or peptide derivative as claimed in claim 1 comprising:
 - (a) the amino acid sequence (I),
 - (b) the amino acid sequence (II),
 - (c) partial regions of the amino acid sequences (I) or/and (II) or/and

- (d) amino acid sequences with a specificity or/and affinity of binding to MHC molecules which is essentially equivalent to that of the amino acid sequences from (a), (b) or/and (c).
3. Peptide or peptide derivative as claimed in claim 1 or 2,
wherein
it has a length of at least 8 amino acids.
 4. Peptide or peptide derivative as claimed in one of the claims 1 to 3,
wherein
it has a length of at least 10 amino acids.
 5. Peptide or peptide derivative as claimed in one of the claims 1 to 3,
wherein
it has a length of up to 25 amino acids.
 6. Peptide or peptide derivative as claimed in one of the claims 1 to 5,
wherein
it carries a marker group.
 7. Peptide-mimetic,
wherein
it has a specificity or/and affinity of binding to MHC molecules which is essentially equivalent to that of a peptide or peptide derivative as claimed in one of the claims 1 to 6.

8. Complex which comprises at least one peptide or peptide derivative as claimed in one of the claims 1 to 6 or a peptide-mimetic as claimed in claim 7 which is bound to a MHC molecule or to a peptide-binding derivative of a MHC molecule.
9. Complex as claimed in claim 8,
wherein
it comprises a MHC class II molecule or a peptide-binding derivative thereof.
10. Complex as claimed in claim 9,
wherein
the MHC class II molecule is of the type DR1, DR2, DR3 or DR4.
11. Complex as claimed in claim 10,
wherein
the MHC class II molecule has the subtype DR B1 0101, DR B1 0301, DR B1 0401, DR B1 0402, DR B1 0404 or DR B1 1601.
12. Complex as claimed in claim 11,
wherein
the MHC class II molecule has the subtype DR B1 0101 or DR B1 0401.
13. Complex as claimed in one of the claims 8 to 12,
wherein
it comprises a recombinant MHC molecule or a peptide-binding derivative thereof.

14. Complex as claimed in claim 13,
wherein
it comprises a soluble peptide-binding derivative
of a MHC molecule.
15. Complex as claimed in one of the claims 8 to 14,
wherein
it carries a marker group.
16. Oligomerized peptide-MHC molecule complex
containing at least 2 MHC molecules or MHC molecule
derivatives which are associated by means of
covalent or non-covalent interactions.
17. Oligomerized complex as claimed in claim 16,
wherein
it contains peptide-MHC molecule complexes cross-
linked by chemical coupling reagents.
18. Oligomerized complex as claimed in claim 16,
wherein
it contains MHC molecules or MHC molecule
derivatives cross-linked by an oligomerized peptide
component with several MHC-binding regions.
19. Oligomerized complex as claimed in claim 16,
wherein
it contains peptide-MHC molecule complexes cross-
linked by antibodies.

20. Oligomerized complex as claimed in one of the claims 16 to 19,
wherein
it contains MHC molecules as defined in one of the claims 9 to 14.
21. Oligomerized complex as claimed in one of the claims 16 to 20,
wherein
it contains at least one peptide or peptide derivative as claimed in one of the claims 1 to 6 or a peptide-mimetic as claimed in claim 7.
22. Pharmaceutical composition,
wherein
it contains a peptide or peptide derivative as claimed in one of the claims 1 to 6, a peptide-mimetic as claimed in claim 7 or/and a complex as claimed in one of the claims 8 to 21 as the active component, if desired in combination with common pharmaceutical additives.
23. Composition as claimed in claim 22,
wherein
it additionally includes an accessory stimulating component.
24. Composition as claimed in claim 23,
wherein
the accessory stimulating component is selected from cytokines or/and the surface antigen B7.

25. Use of a pharmaceutical composition as claimed in one of the claims 22 to 24 for the production of an agent for the diagnosis of diseases or of a predisposition to diseases which influence the immune system or of tumour diseases or of a predisposition to tumour diseases.
26. Use as claimed in claim 25 for the production of an agent for the diagnosis of autoimmune diseases or of a predisposition to autoimmune diseases.
27. Use as claimed in claim 25 or 26 for the production of an agent for the diagnosis of diabetes or of a predisposition to diabetes.
28. Method for the determination of a specific T cell subpopulation,
wherein
a sample containing T cells is brought into contact with a peptide or peptide derivative as claimed in one of the claims 1 to 6, a peptide-mimetic as claimed in claim 7 or/and a complex as claimed in one of the claims 8 to 21 and the reaction of T cells in the sample with the peptide or complex is determined.
29. Method as claimed in claim 28,
wherein
the reaction of the T cells is determined by FACS analysis using a fluorescent-labelled peptide or complex.

30. Method as claimed in claims 28 to 29,
wherein
before and/or after contact of the T cells with the peptide or the complex, a selection for pre-activated T cells is carried out.
31. Use of a pharmaceutical composition as claimed in one of the claims 22 to 24 for the production of an agent for the therapy or prevention of diseases which influence the immune system.
32. Use as claimed in claim 31 for the production of an agent for the therapy or prevention of autoimmune diseases.
33. Use as claimed in claim 31 or 32 for the production of an agent for the therapy or prevention of diabetes.
34. Use of a peptide or peptide derivative as claimed in one of the claims 1 to 6, a peptide-mimetic as claimed in claim 7 or a complex as claimed in one of the claims 8 to 21 for the production of an antigen, in particular of an immunogen or tolerogen.
35. Process for the isolation of a specific T cell subpopulation,
wherein
a sample containing T cells is brought into contact with a peptide or peptide derivative as claimed in one of the claims 1 to 6, with a peptide-mimetic as

claimed in claim 7 or with a complex as claimed in one of the claims 8 to 21, the T cells reacting with the peptide or complex are identified and if desired are separated from other T cells.

36. Process as claimed in claim 35,
wherein
before or/and after contact of the T cells with the peptide or the complex, a selection for pre-activated T cells is carried out.
37. Use of T cells isolated according to the process as claimed in claim 35 or partial structures thereof for the production of an antigen.
38. Use as claimed in claim 37,
wherein
the T cells or partial structures thereof are reinjected into the patients from which they were originally derived.
39. Use as claimed in claim 38,
wherein
inactivated T cells are reinjected.
40. Use as claimed in claim 39,
wherein
T cells capable of division are reinjected.
41. Antibody against a peptide or peptide derivative as claimed in one of the claims 1 to 6, a peptide-mimetic as claimed in claim 7 or a complex as claimed in one of the claims 8 to 21, obtainable by

immunization with the peptide, peptide derivative, peptide-mimetic or complex and isolation of an antibody produced by the immunization.

42. Anti-idiotypic antibody against an antibody as claimed in claim 41, obtainable by immunization with the antibody against the peptide, peptide derivative or peptide-mimetic or the complex and isolation of an anti-idiotypic antibody produced by the immunization.
43. T cell which reacts with a peptide or peptide derivative as claimed in one of the claims 1 to 6, with a peptide-mimetic as claimed in claim 7 or with a complex as claimed in one of the claims 8 to 15 or 21.
44. T cell as claimed in claim 43 which is derived from the T cell line 6/7 (DSM ACC2172) or has an equivalent T cell receptor binding specificity.
45. T cell as claimed in claim 43 which is derived from the T cell line 6/10 (DSM ACC2173) or has an equivalent T cell receptor binding specificity.

ABSTRACT

The invention concerns autoreactive peptides, peptide-MHC complexes, T cell subpopulations that react thereto as well as diagnostic and therapeutic applications of these compounds.

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Fig. 1

I-L-I-K-C-D-E-R-G-K-M-I-P-S

L-G-I-G-T-D-S-V-I-L-I-K-C-D

L-A-F-L-Q-D-V-M-N-I-L-L-Q-Y

Y-D-L-S-Y-D-T-G-D-K-A-L-Q-C

Fig. 2

V-S-Y-Q-P-L-G-D-K-V-N-F-F-R
L-A-A-D-W-L-T-S-T-A-N-T-N-M
L-L-Y-G-D-A-E-K-P-A-E-S-G-G
V-N-Y-A-F-L-H-A-T-D-L-L-P-A
L-L-Q-Y-V-V-K-S-F-D-R-S-T-K
F-T-Y-E-I-A-P-V-F-V-L-L-E-Y
L-E-Y-V-T-L-K-K-M-R-E-I-I-G
N-M-Y-A-M-M-I-A-R-F-K-M-F-P
K-I-W-M-H-V-D-A-A-W-G-G-G-L
W-G-G-G-L-L-M-S-R-K-H-K-W-K
E-G-Y-E-M-V-F-D-G-K-P-Q-H-T
R-Y-F-N-Q-L-S-T-G-L-D-M-V-G
W-L-T-S-T-A-N-T-N-M-F-T-Y-E
T-A-N-T-N-M-F-T-Y-E-I-A-P-V
L-V-S-A-T-A-G-T-T-V-Y-G-A-F
Y-I-P-P-S-L-R-T-L-E-D-N-E-E
V-I-S-N-P-A-A-T-H-Q-D-I-D-F

Fig. 3

| Proliferation assay pat 6/7 with peptides

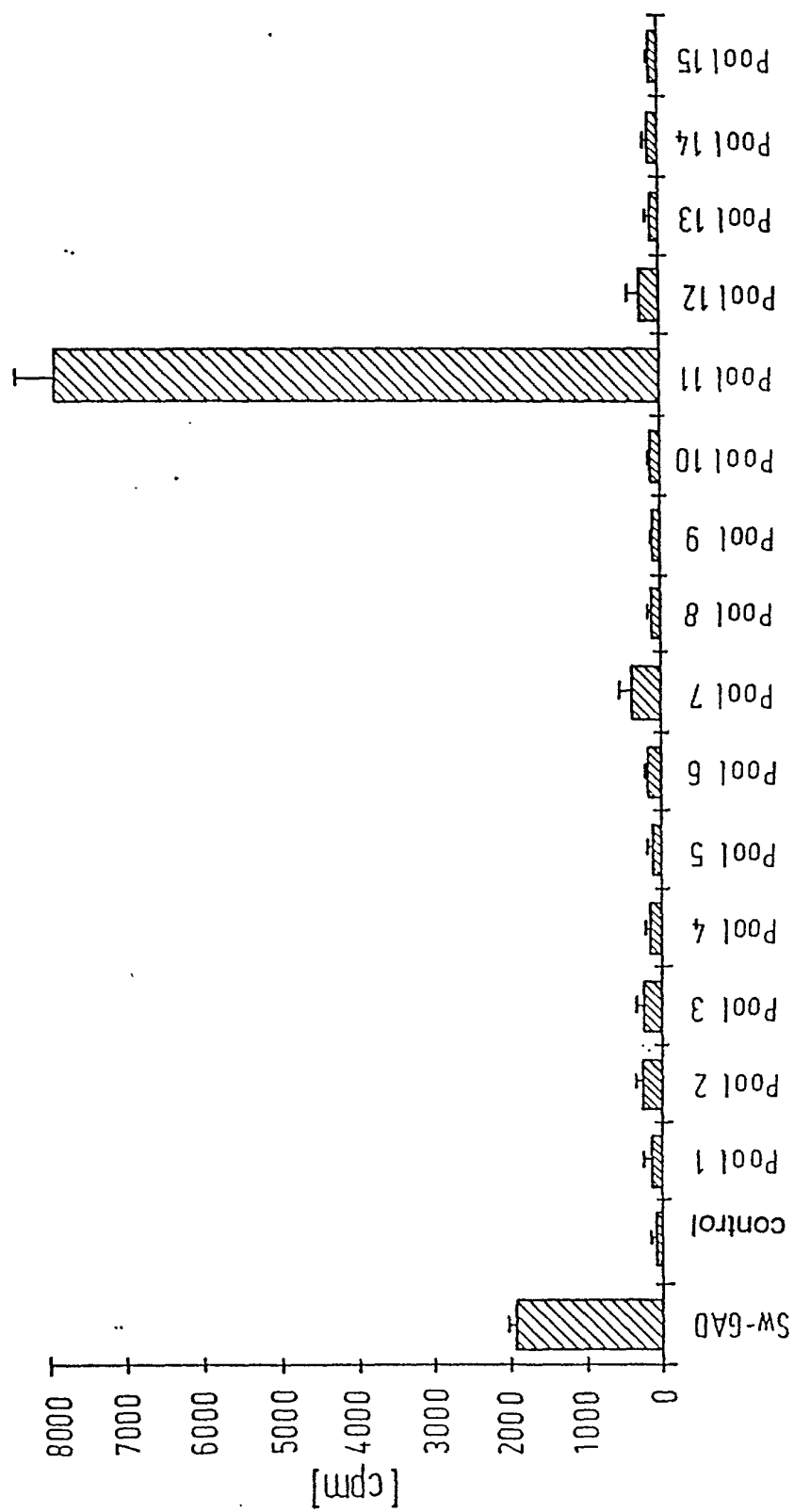


Fig. 4

Proliferation assay pat 6/10 with peptides

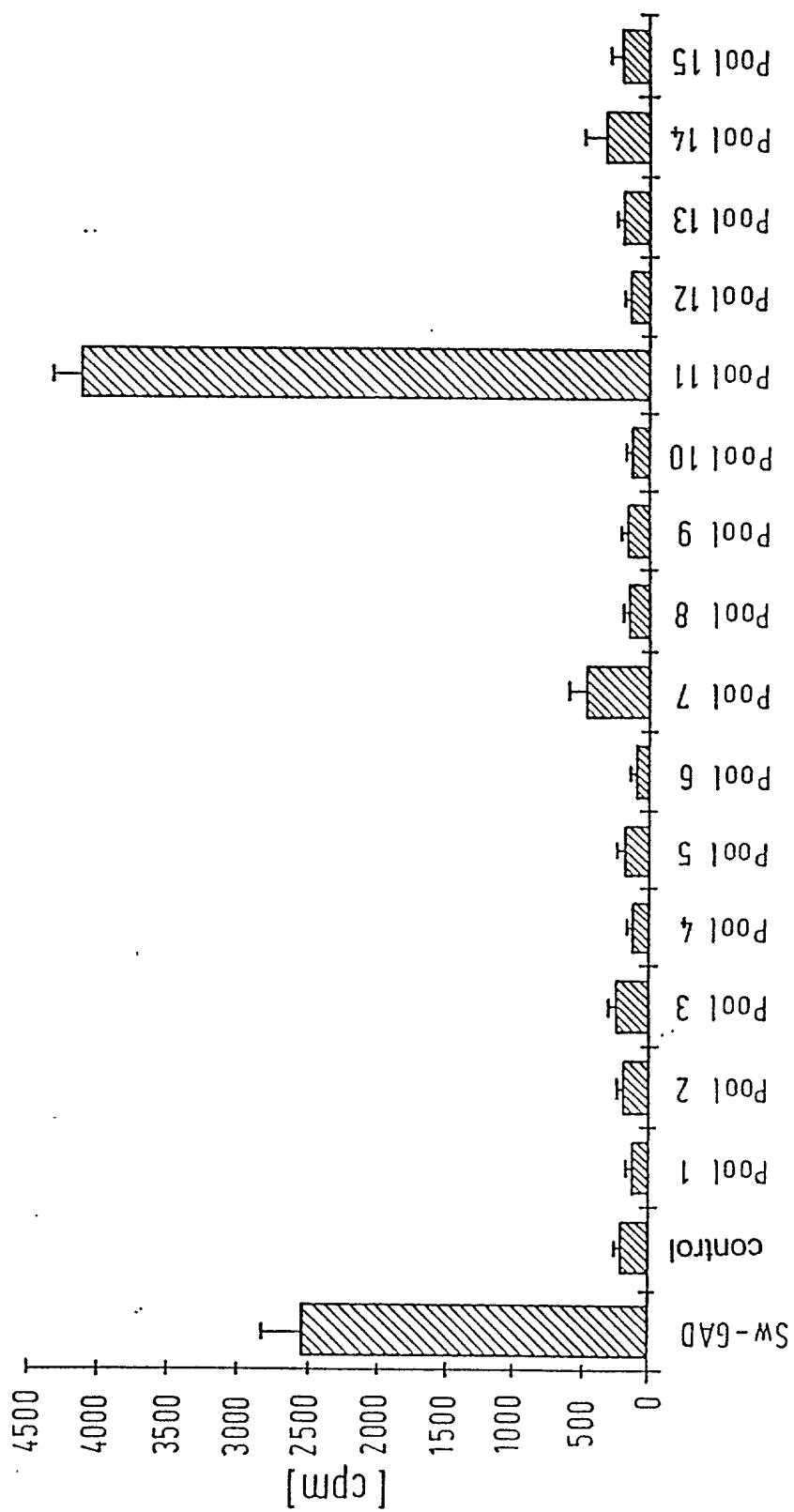


Fig. 5

Proliferation assay pat. 6/7 with individual peptides from pool 7 and 11

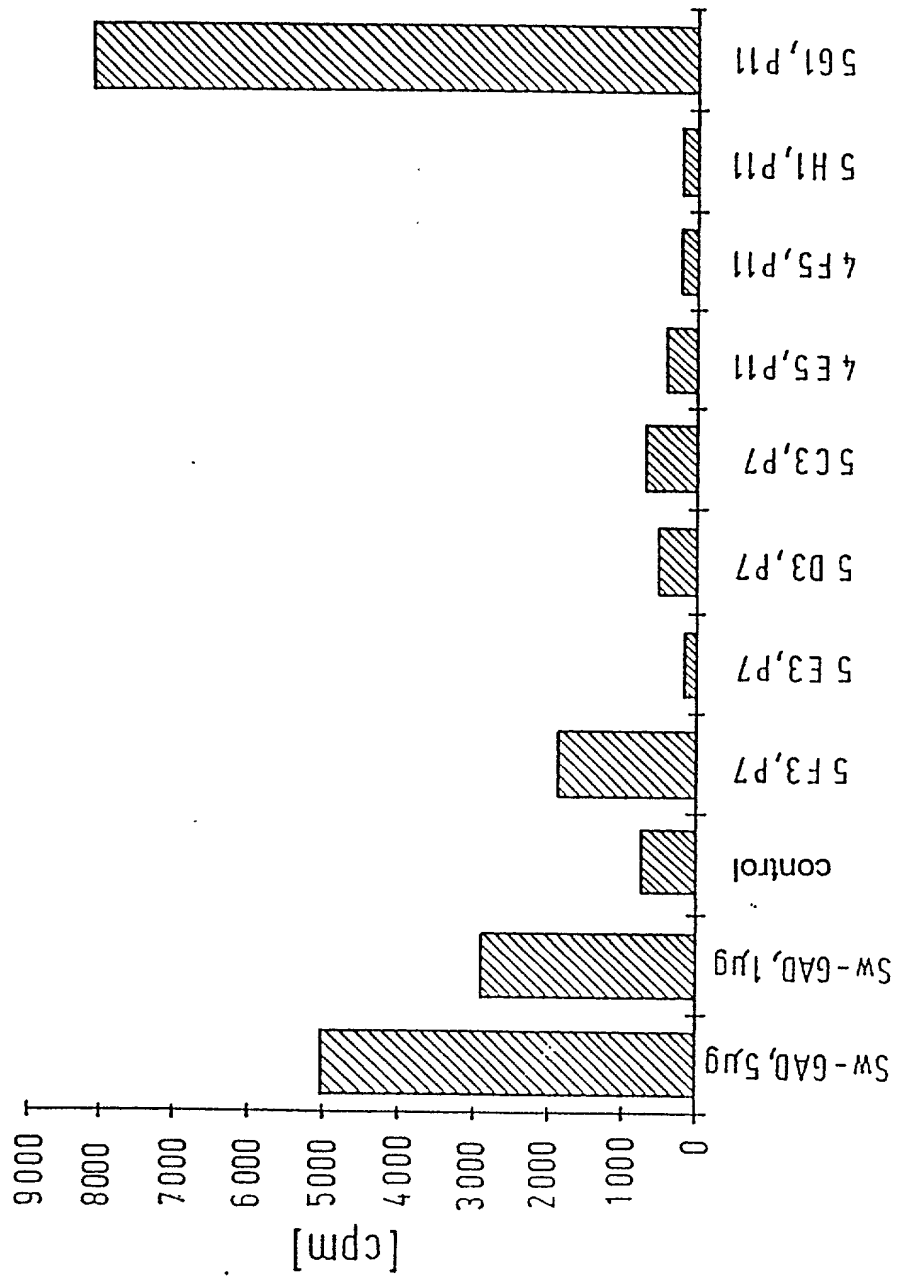
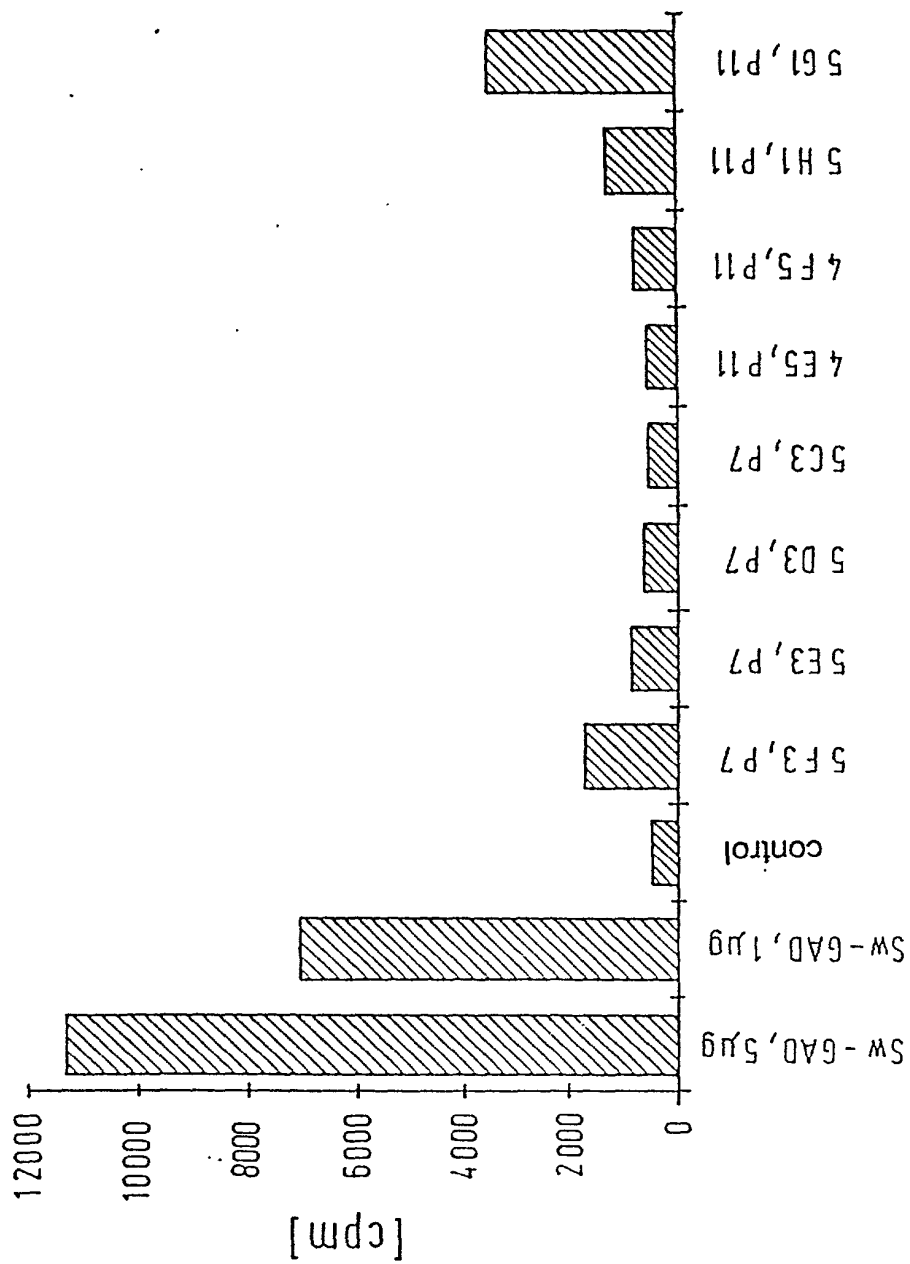


Fig. 6

Proliferation assay pat. 6/10 with individual peptides from pool 7 and 11



Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
(Insert Title) ANTIGEN-SPECIFIC, ACTIVATED T LYMPHOCYTES, DETECTION AND USE
the specification of which

(Check one of blocks 1, 2 or 3. See note A on back of this page)

1. ☐ is attached hereto.
2. ☐ was filed on _____ as International PCT Application Serial No. _____ and was amended on _____ (if applicable)
3. ☒ was filed on January 18, 1995 _____ as U.S. Application Serial No. 08/374,468 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed:

(List prior foreign applications. See note B on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
	<u>P 44 01 629 8</u>	<u>DE</u>	<u>20/1/94</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
	<u>P 44 03 522 5</u>	<u>DE</u>	<u>4/2/94</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
	<u>P 44 18 091 8</u>	<u>DE</u>	<u>24/5/94</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
	_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
	_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

(See Note C on back of this page)

☐ See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	_____	_____	_____
	_____	_____	_____

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Michael G. Gilman, Reg. No. 19,114; Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Sharon N. Klesner, Reg. No. 36,335, and John R. Fuisz, Reg. No. 37,327.

Please direct all communications to the following address: **NIKAIDO, MARMELESTEIN, MURRAY & ORAM**
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note D on back of this page)

Full name of sole or first inventor Josef ENDL
Inventor's signature *Josef Endl* 31.3.95 Date
Residence Ulmenstraße 12, Federal Republic of Germany, 82362 Weilheim
Citizenship German
Post Office Address D-82362 Weilheim, Federal Republic of Germany

Full name of second joint inventor, if any Peter STAHL

Inventor's signature Peter Stahl

April 16, 1995

Date

Residence Hirtenstraße 12, Federal Republic of Germany, 82347 Bernried

Citizenship German

Post Office Address D-82347 Bernried, Federal Republic of Germany

Full name of third joint inventor, if any Winfried ALBERT

Inventor's signature Winfried Albert

April 16, 1995

Date

Residence Hauptstraße 16a, Federal Republic of Germany, 82390 Eberfing

Citizenship Austrian

Post Office Address D-82390 Eberfing, Federal Republic of Germany

Full name of fourth joint inventor, if any Guenther-Gerhard JUNG

Inventor's signature Guenther-Gerhard Jung

April 15, 1995

Date

Residence Ob der Grafenhalde 5, Federal Republic of Germany, 72076 Tübingen

Citizenship German

Post Office Address D-72076 Tübingen, Federal Republic of Germany

Full name of fifth joint inventor, if any Dolores I. SCHENDEL

Inventor's signature Dolores I. Schendel

April 26, 1995

Date

Residence Hans-Sachs-Straße 123, Federal Republic of Germany, 80469 München

Citizenship United States of America

Post Office Address D-80469 München, Federal Republic of Germany

Full name of sixth joint inventor, if any Edgar MEINL

Inventor's signature Edgar Meinl

April 28, 1995

Date

Residence Stieglitz Weg 11, Federal Republic of Germany, 82152 Krailling

Citizenship German

Post Office Address D-82152 Krailling, Federal Republic of Germany

Full name of seventh joint inventor, if any Klaus DORNMAIR

Inventor's signature Klaus Dornmair

4 May 95

Date

Residence Auenstraße 1a, Federal Republic of Germany, 82275 Emmering

Citizenship German

Post Office Address D-82275 Emmering, Federal Republic of Germany

Full name of eighth joint inventor, if any

Inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of ninth joint inventor, if any

Inventor's signature

Date

Residence

Citizenship

Post Office Address

66977 507160

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

ENDL, et al.

Serial Number: Not Yet Assigned

Filed: November 15, 1999

For: ANTIGEN-SPECIFIC ACTIVATED T LYMPHOCYTES, DETECTION AND USE

REQUEST TO USE PREVIOUSLY FILED SEQUENCE LISTING
UNDER MPEP 2422.05

Assistant Commissioner
of Patents
Washington, D.C. 20231

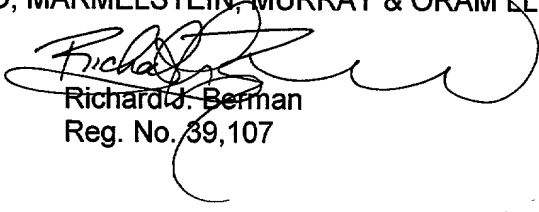
November 16, 1999

Sir:

The paper copy of the sequence Listing in this divisional of 09/343,406, filed June 30, 1999 which is a divisional of 08/967,242, which is a continuation of 08/374,468, is identical to the computer readable copy of the Sequence Listing filed in parent application 08/374,468 filed July 17, 1996. In accordance with 37 CFR 1.821(e), please use the computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the instant application. A paper copy of the Sequence Listing is included in the Preliminary Amendment for incorporation into the specification.

If any fees are due with connection to the filing of this paper, please charge to our Deposit Account No. 14-1060.

Respectfully submitted,
NIKAIDO, MARMELESTEIN, MURRAY & ORAM LLP


Richard J. Berman
Reg. No. 39,107

Atty. Case No. P564-9035
Metropolitan Square
655 Fifteenth Street, N.W.
G Street Lobby - Suite 330
Washington, D.C. 20005-5701
RJB:ccd



09/441061

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ENDL, Josef
STAHL, Peter
ALBERT, Winfried
JUNG, Guenther-Gerhard
SCHENDEL, Dolores J.
MEINL, Edgar
DORNMAIR, Klaus

(ii) TITLE OF INVENTION: ANTIGEN-SPECIFIC, ACTIVATED T
LYMPHOCYTES, DETECTION AND USE

(iii) NUMBER OF SEQUENCES: 39

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Nikaido, Marmelstein, Murray & Oram LLP
(B) STREET: 655 Fifteenth Street N.W. Suite 330
(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: U.S.A.
(F) ZIP: 20005-5701

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/374,468
(B) FILING DATE: 18-JAN-1995
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: DE P 44 01 629.8
(B) FILING DATE: 20-JAN-1994

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: DE P 44 03 522.5
(B) FILING DATE: 04-FEB-1994

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: DE P 44 18 091.8
(B) FILING DATE: 24-MAY-1994

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Murray, Robert B.
- (B) REGISTRATION NUMBER: 22,980
- (C) REFERENCE/DOCKET NUMBER: P564-U5002

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (202) 638-5000
- (B) TELEFAX: (202) 638-4810

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: -
- (D) TOPOLOGY: linear -

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Xaa in position 1 is an optional sequence selected from 1 to 10 amino acids."

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /note= "Xaa in position 6 is Thr or Glu."

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /note= "Xaa in position 8 is an optional sequence selected from 1 to 8 amino acids."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Xaa Pro Glu Val Lys Xaa Lys Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid

- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Met Ala Ala Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser
1 5 10 15
His Phe Ser Leu Lys Lys Gly Ala Ala
 20 25

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Arg Gly Lys Met Ile Pro Ser Asp Leu Glu Arg Arg Ile Leu Glu
1 5 10 15
Ala Lys Gln Lys
 20

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser His Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly	Met	Ala	Ala	Leu	Pro	Arg	Leu	Ile	Ala	Phe	Thr	Ser	Glu	His	Ser
1				5				10						15	
His Phe Ser Leu															
20															

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala	Leu	Pro	Arg	Leu	Ile	Ala	Phe	Thr	Ser	Glu	His	Ser	His	Phe	Ser
1				5				10					15		
Leu Lys Lys Gly															
20															

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Arg Leu Ile Ala Phe Thr Ser Glu His Ser His Phe Ser Leu Lys Lys
1 5 10 15
Gly Ala Ala Ala
20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Pro Glu Val Lys Glu Lys Gly Met Ala Ala Leu Pro Arg Leu Ile Ala
1 5 10 15
Phe Thr Ser Glu
20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Ala Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser His Phe
1 5 10 15
Ser Leu

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Ala Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser His Phe
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Ala Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Ala Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 14 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Met Ala Ala Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Met Ala Ala Leu Pro Arg Leu Ile Ala Phe Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser His Phe Ser Leu
1 5 10 15

Lys Lys

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Arg Leu Ile Ala Phe Thr Ser Glu His Ser His Phe Ser Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser His Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Leu Pro Arg Leu Ile Ala Phe Thr Ser Glu His Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ile Leu Ile Lys Cys Asp Glu Arg Gly Lys Met Ile Pro Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Gly Ile Gly Thr Asp Ser Val Ile Leu Ile Lys Cys Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Ala Phe Leu Gln Asp Val Met Asn Ile Leu Leu Gln Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Tyr Asp Leu Ser Tyr Asp Thr Gly Asp Lys Ala Leu Gln Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Val Ser Tyr Gln Pro Leu Gly Asp Lys Val Asn Phe Phe Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Leu Ala Ala Asp Trp Leu Thr Ser Thr Ala Asn Thr Asn Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Leu Leu Tyr Gly Asp Ala Glu Lys Pro Ala Glu Ser Gly Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Val Asn Tyr Ala Phe Leu His Ala Thr Asp Leu Leu Pro Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Leu Leu Gln Tyr Val Val Lys Ser Phe Asp Arg Ser Thr Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Phe Thr Tyr Glu Ile Ala Pro Val Phe Val Leu Leu Glu Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Leu Glu Tyr Val Thr Leu Lys Lys Met Arg Glu Ile Ile Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Asn Met Tyr Ala Met Met Ile Ala Arg Phe Lys Met Phe Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Lys Ile Trp Met His Val Asp Ala Ala Trp Gly Gly Gly Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Trp Gly Gly Gly Leu Leu Met Ser Arg Lys His Lys Trp Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Glu Gly Tyr Glu Met Val Phe Asp Gly Lys Pro Gln His Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Arg Tyr Phe Asn Gln Leu Ser Thr Gly Leu Asp Met Val Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Trp Leu Thr Ser Thr Ala Asn Thr Asn Met Phe Thr Tyr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Thr Ala Asn Thr Asn Met Phe Thr Tyr Glu Ile Ala Pro Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Leu Val Ser Ala Thr Ala Gly Thr Thr Val Tyr Gly Ala Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Tyr Ile Pro Pro Ser Leu Arg Thr Leu Glu Asp Asn Glu Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

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Val Ile Ser Asn Pro Ala Ala Thr His Gln Asp Ile Asp Phe
1 5 10